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Competitive immunoassay of phenobarbital by microchip electrophoresis with laser induced fluorescence detection

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ABSTRACT

A microchip electrophoresis method with laser induced fluorescence detection was developed for the immunoassay of phenobarbital. The detection was based on the competitive immunoreaction between analyte phenobarbital and fluorescein isothiocyanate (FITC) labeled phenobarbital with a limited amount of antibody. The assay was developed by varying the borate concentration, buffer pH, separation voltage, and incubation time. A running buffer system containing 35 mM borate and 15 mM sodium dodecyl sulfate (pH 9.5), and 2800 V separation voltage provided analysis conditions for a high-resolution, sensitive, and repeatable assay of phenobarbital. Free FITC-labeled phenobarbital and immunocomplex were separated within 30 s. The calibration curve for phenobarbital had a detection limit of 3.4 nM and a range of 8.6–860.0 nM. The assay could be used to determine the phenobarbital plasma concentration in clinical plasma sample.

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1. Introduction

Phenobarbital is frequently used anti-epileptic drug. During epileptic therapies, low concentrations of phenobarbital can cause break-through seizures, while the concentrations above the therapeutic range may result in toxic symptoms [1–3]. Thus, by monitoring the plasma phenobarbital concentration, the dose administered can be adjusted to the optimal level for effective therapeutic control but with minimal side effects such as neurological toxicity.

Historically, there are several methods for determining phenobarbital concentration in body fluids. These methods include mainly high-performance liquid chromatography [4–11], gas chromatography [12–14] and capillary electrophoresis [15–17]. However, as a result of the increase in the number of determinations done for antiepileptic drugs, immunoassay has become the most common method for routine therapeutic drug monitoring because of its simplicity, reliability and speed. Besides traditional immunoanalytical methods, fluorescence polarization immunoassay [18], capillary electrochemical enzyme immunoassay [19], electrochemical immunoassay [20,21], time-resolved fluoroimmunoassay [22] and carbonylmetalloimmunoassay [23–25] are also available for the determination of phenobarbital in plasma.

Microchip electrophoresis has been proven to be a powerful tool for the separation of various chemical species from small molecules to macromolecules such as DNA and protein [26,27]. Microchip electrophoresis combined with immunoassay can separate rapidly the free antigen and antibody from the bound antigen and antibody. Microchip electrophoretic immunoassay offers many advantages including simplification of analytical procedures, reduction of assay time and lower consumption of samples and reagents when compared with conventional immunoassay techniques. Microchip electrophoretic immunoassay technique has been applied for the determination of cortisol, theophylline, hormones, peptides, proteins and 2,4,6-trinitrotoluene [28–36].

Microchip electrophoretic immunoassay technique is very useful for carrying out determinations of drugs in clinical plasma sample. In this work, a microchip electrophoretic immunoassay method with laser induced fluorescence detection was developed for the determination of phenobarbital. Conditions for immunoreaction and microchip electrophoresis separation were studied. And the quantitative measurements of phenobarbital in plasma from epileptic patients have been demonstrated. The results indicated that the proposed microchip electrophoretic immunoassay method with laser induced fluorescence detection has a promise for sim-

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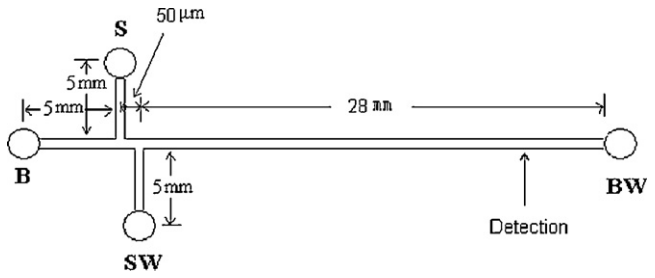

Fig. 1. The layout and dimensions of the glass/PDMS hybrid microchip used in this work. S: sample reservoir; SW: sample waste reservoir; B: buffer reservoir; BW: buffer waste reservoir.

ple, rapid, sensitive, and low-cost clinical assays of therapeutic drugs.

2. Experimental

2.1. Reagents and chemicals

All the reagents and chemicals used in this work were of analytical grade. Polyclonal anti-phenobarbital antibody (Ab), fluorescein isothiocyanate (FITC)-labeled phenobarbital (Ag*), phenobarbital (Ag) were purchased from Abbott Labs. (Chicago, IL, USA). Water was purified by employing a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA), and used throughout the work. All standard solutions were prepared with the physiological buffer solution (PBS) consisted of 0.135 M NaCl and 0.02 M NaH_2PO_4–NaOH (pH 7.4). The electrophoretic buffer was 35 mM borate solution (pH 9.5, adjusted with 1 M NaOH solution) containing 15 mM SDS. All solutions were filtered through 0.22 μm membrane filters before use.

2.2. Apparatus and microfluidic chip

The microchip electrophoresis-confocal laser induced fluorescence detection system with 473 nm semiconductor laser was built by the Shandong Normal University. A multi-terminal high voltage power supply, variable in the range of 0–6000 V, was used for sample injection and microchip electrophoresis separation. The output signal was recorded and processed with a computer using a chromatography data system (Zhejiang University Star Information Technology, Hangzhou, China). A home-made glass/PDMS hybrid microfluidic chip was used for the separation of sample. The fabrication of the glass/PDMS microchip was performed according to the procedure described previously [37]. The layout and dimensions of the chip is shown in Fig. 1. All channels etched in glass substrates were 20 μm deep and 50 μm wide. The distance between sampling channel and sample waste channel (center-to-center distance) was 50 μm. The effective separation channel length was 2.1 cm. All reservoirs were 4 mm in diameter and 2 mm deep.

2.3. Sample preparation

The human plasma samples were obtained from five epileptic patients. The human blood samples were collected after 10 h ingesting therapeutic amount phenobarbital, which were centrifuged at 2000×*g* for 15 min immediately to obtain the plasma. The preparation of the plasma sample was similar to the procedure described by Rukhadze et al. [7]. A portion (100 μL) of human plasma was diluted with 100 μL of 0.3 M hydrochloric acid and 1.0 mL of chloroform. The mixture was shaken vigorously for 2 min and centrifuged at 3000×*g* for 10 min. The organic layer was transferred into another 1.5 mL vial and dried with a nitrogen stream for 40 min. The residue was dissolved in 100 μL of PBS (pH 7.4). The solution was vortexed and kept at −20 °C. The sample solution was diluted 1000-fold before analysis.

2.4. Immunoreaction procedure

Fifteen microlitres of standard phenobarbital or sample solution were mixed with 15 μL of 200 nM FITC-labeled phenobarbitals and 15 μL of 100 nM polyclonal anti-phenobarbital antibody in a 0.5 mL microcentrifuge tube. This solution was incubated at 37 °C for 10 min. For preparation of standard calibration curves, the concentrations of phenobarbital standard solutions were varied from 5.0 nM to 900 nM, and the concentrations of FITC-labeled phenobarbital and polyclonal anti-phenobarbital antibody were maintained constant at 200 nM and 100 nM, respectively.

2.5. Procedures for separation and detection

Between two consecutive runs, the microchannels were rinsed sequentially with 0.1 M NaOH, water and electrophoretic buffer for 5 min each. Prior to electrophoresis, all reservoirs were filled with the electrophoretic buffer. Vacuum was applied to the reservoir BW in order to fill the separation channel with the electrophoretic buffer. Then, the electrophoretic buffer solution in reservoir S was replaced by sample solution. For loading the sample solution, a set of electrical potentials were applied to the four reservoirs: reservoir S at 900 V, reservoir B at 250 V, reservoir BW at 400 V, reservoir SW at grounded. The sample solution was transported from reservoir S to SW in pinched mode. After 10 s, potentials were switched to reservoir B, S and SW at 2800, 720 and 720 V, respectively, while reservoir BW was grounded for separation and detection.

3. Results and discussion

3.1. Optimization of separation conditions

The separation of free labeled antigen and the immunocomplex is a key step in all microchip electrophoresis-based competitive immunoassays. To achieve a well separation, the parameters affecting the separation, such as concentration of electrolyte, pH of electrophoretic buffer, SDS concentration and applied voltage were optimized.

3.1.1. Effect of SDS concentration

In microchip electrophoretic immunoassay, immune reagents were easily adsorbed on the surface of microchannel. Adding surfactants into electrophoretic buffer has been proven to be an effective method to avoid the surface adsorption [38]. In this work, an anionic surfactant SDS was used as an additive, and the influence of SDS concentration ranging from 5 mM to 25 mM on the separation was investigated. The results indicate that the resolution of Ag* and the Ag*–Ab complex increased with the increase of SDS concentration, however, the migration times of both Ag* and the Ag*–Ab complex also slightly prolonged with increase of SDS concentration. By considering the resolution and analysis time, a SDS concentration of 15 mM was selected for the following experiments.

3.1.2. Effect of the concentration and pH of electrophoretic buffer

The electrophoretic buffer pH is an important parameter in microchip electrophoresis-based immunoassays, because it affected significantly the characteristics of channel surfaces, and the effective electric charge of the analytes. In this work, the electrophoretic buffers at different pH values were evaluated. It was noted that optimum resolution was obtained with borate buffer at pH 9.5 (Fig. 2). The effect of the borate concentration on resolution was also examined. It was found that the resolution of Ag* and the

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